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Structure and Function of a Special Tissue in the Female Genital Ducts of the Chinese Freshwater Crab *Eriocheir sinensis*

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Abstract. The histological anatomy of the genital ducts of adult females of *Eriocheir sinensis* was studied before and after copulation, and during and after egg-laying. A strongly basophilic valve-like tissue was discovered at the junction of the spermatheca and the oviduct. This tissue prevents communication between the spermatheca and the oviduct except during oviposition. At this time, it functions as a valve, allowing ripe eggs out of the oviduct and preventing sperm from entering the oviduct during and after egg-laying. These findings suggest that the actual site of gamete contact in *E. sinensis* is within the spermatheca, instead of in the lumen of the ovary or in the oviduct. The presence of the valve-like tissue assures that the ripe eggs collected from the ovary during egg-laying are unfertilized. This observation is of great importance for obtaining unfertilized ripe eggs in studies of artificial fertilization (*in vitro*) and hybridization. The valve-like tissue has not been described in other brachyurans, and this genital duct should be classified as new for the Brachyura.

Introduction

The goal of this study was to define the actual site of fertilization in the Chinese freshwater crab, *Eriocheir sinensis*, in preparation for artificial fertilization (*in vitro*). This crab is widely distributed in fresh and brackish waters in southeastern China and has great economic value in the country.

The female reproductive system of the Brachyura, with the exception of two superfamilies, consists of a series of ducts leading from the ovary to the exterior of the animal. These ducts are composed of four regions: oviduct, spermatheca, vagina, and vulva (Hartnoll, 1968). During copulation, the male transfers its spermatophores into the spermatheca of the female; therefore, fertilization in the Brachyura is generally accepted as being internal. But what is the actual site of this fertilization? And what is meant by "internal fertilization" in the Brachyura? These two questions have not yet been answered conclusively.

Early reports were contradictory. Binford (1913) suggested that in *Menippe mercenaria*, fertilization occurred in the lumen of the ovary, because spermatozoa were found on the surface of the ripe eggs, and many could develop into embryos. Spalding (1942), Cheung (1966), and Goudeau (1982) reported that fertilization of *Carcinus maenas* occurs in the lumen of the ovary or within the oviduct. On the other hand, studies of *Portunus sanguinolentus* (by Ryan, 1967) and *Libinia emarginata* (by Hinsch, 1971) suggested that the spermatozoan contacts the membrane of the ripe egg internally, and that the remaining processes in fertilization occur outside the body of the female. This suggestion agrees with that of Yonge (1937).

In *E. sinensis*, we found a valve-like tissue within the spermathecal wall which is connected to the oviduct. Except during oviposition, this valve-like tissue prevents communication between the oviduct and the spermatheca. During egg-laying, the tissue functions as a valve, freeing eggs and preventing spermatozoa from entering the oviduct. Thus, we will comment on the expression "internal fertilization" as it pertains to *E. sinensis* and other brachyurans.

Materials and Methods

Specimens of the Chinese freshwater crab, *Eriocheir sinensis*, were obtained from Yang Qin Lake in Jiangsu

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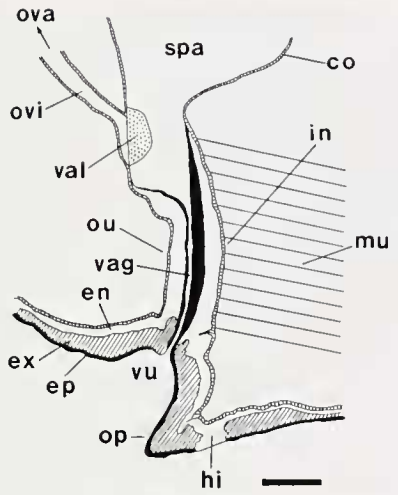

Figure 1. Schematic illustration of the longitudinal section of the genital ducts of the female *Eriocheir sinensis*. Abbreviations: (co) columnar epithelium; (en) endocuticle; (ex) exocuticle; (ep) epicuticle; (hi) hinge; (in) inner wall; (mu) muscles; (op) operculum; (ou) outer wall; (ova) ovary; (ovi) oviduct; (spa) spermatheca; (vag) vagina; (val) valve-like tissue; (vu) vulva. Bar = 1 mm.

Province, China, in November 1986 during the crab's spawning migration season. The maximum carapace widths of the specimens used in this study were 7–8 cm. Some specimens were used immediately after they arrived at the laboratory (Faculty of Fisheries, Hokkaido University, Hakodate, Japan); these were at the germinal vesicle stage. The remaining specimens were maintained for about two months in individual compartments of a well-aerated, closed circulating seawater system (28‰ S, 20°C). Female genital ducts, in various stages—before and after copulation, during egg-laying, and one day and four days after egg-laying—were excised carefully and fixed in Bouin's solution for histological observations. Sections (8–10 μm) were made by the standard paraffin method and stained with Delafield's haematoxylin and eosin.

Results

Structure of the female genital ducts and the valve-like tissue

In adults of *Eriocheir sinensis*, we found that the female genital ducts have four regions: oviduct, spermatheca, vagina, and vulva (Fig. 1).

The oviduct, a short, tube-like passage connecting the ovary and the spermatheca, is about 4.1 mm long. The opening of the oviduct leading to the spermatheca is on the epithelium of the spermatheca, just above the outer wall of the vagina. The undulant wall of the oviduct is composed of a columnar epithelium. The cavity of the oviduct is full of basophilic material in colloidal form.

The spermatheca is ovoid, about 19 mm high and 8 mm wide. It consists of a single crumpled layer of columnar epithelium; its cavity is filled with a basophilic colloidal substance. The portion of the spermathecal cavity nearest the vagina sharply tapers downward, its narrow end continuous with the cavity of the vagina. The vagina, about 3 mm long, is formed by two cuticular walls; one face (inner wall) is invaginated into a concavity of the other (outer wall) (Fig. 2). Muscles run diagonally from the inner wall to the sternum. The opening of the vagina (*i.e.*, the vulva) is on the sternite of the sixth thoracic segment. The vulva is characterized by the presence of an operculum—the continuation of the inner wall of the vagina. The operculum can be opened and closed by contracting and relaxing the muscles of the inner wall during copulation or egg-laying.

A strongly basophilic tissue (hereafter referred to as the valve-like tissue) can be found at the opening of the spermatheca leading to the oviduct (Fig. 1; Fig. 3A); it is about 1.1 mm high and 0.4 mm wide. Because the border of the valve-like tissue is connected to the epithelium of the opening, it prevents communication between the oviduct and the spermatheca. The tissue is composed of a mass of cells in which no nuclear division is observed; thus, it appears to originate from epithelial cells somewhere nearby.

Under light microscopy, the tissue appeared to be a syncytium because no cell membranes were observed. Most of the nuclei are nearly oval, with a long axis of about 5.6 μm and a short axis of about 3.5 μm; the directions of the long axes are random (Fig. 3B). In contrast, the nuclei in the middle of this tissue are somewhat condensed and spindle-shaped, with a long axis of about 7.3 μm and a short axis of about 2.0 μm; all the long axes of these nuclei point toward the cavity of the spermatheca (Fig. 3C). The nuclei within the middle part of that surface of the tissue facing the spermathecal cavity appeared to be pycnotic (Fig. 3D). This observation suggests that old nuclei are displaced through the middle part of the tissue into the cavity of the spermatheca.

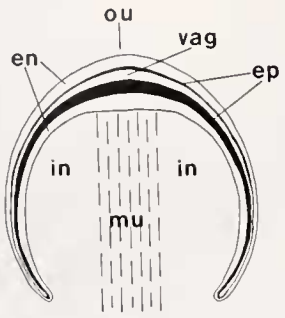

Figure 2. Schematic illustration of a transverse section of the vagina showing its concave shape. Abbreviations: (en) endocuticle; (ep) epicuticle; (in) inner wall; (mu) muscles; (ou) outer wall; (vag) vagina.

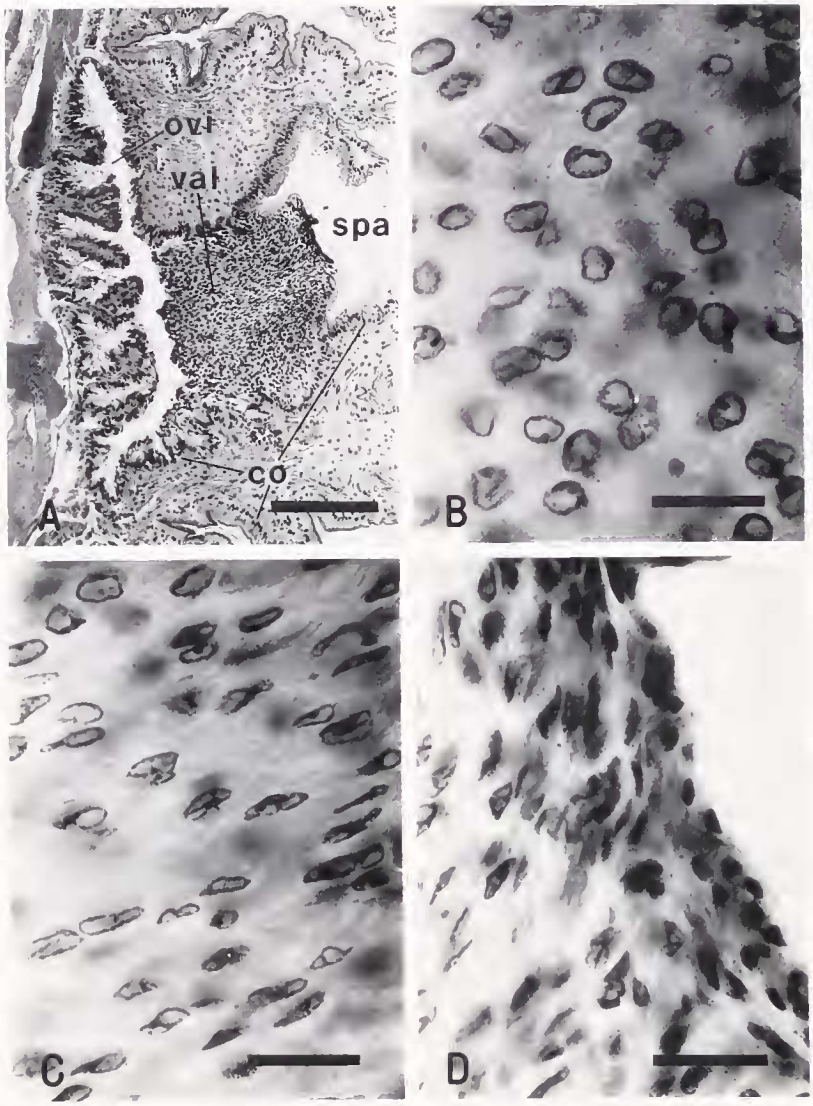

Figure 3. Transverse sections from the middle of the valve-like tissue and oviduct before copulation. A: Whole figure of the valve-like tissue. B: Most of the nuclei of the tissue are oval and the directions of long axes are at random. C: The nuclei in the middle of the tissue are somewhat condensed and spindle-shaped, all the long axes of these nuclei point toward the cavity of the spermatheca. D: The nuclei within the middle part of that surface of the tissue facing the spermathecal cavity appear to be pycnotic. Abbreviations: (co) columnar epithelium; (ovi) oviduct; (spa) spermatheca; (val) valve-like tissue. Bar (A) = 200 μm. Bar (B, C, D) = 20 μm.

Structural changes in the valve-like tissue and the oviduct

Five hours after copulation. Spermatophores and free spermatozoa introduced during copulation swell the spermatheca to about three times its pre-copulatory size. The valve-like tissue is a bit flattened due to the pressure of the seminal fluid (Fig. 4A). Besides these, no other changes in the oviduct were observed.

During egg-laying. Changes occur in both the oviduct and the valve-like tissue. The undulant surface of the oviduct straightens, and its circumference expands to some degree. The valve-like tissue is perforated by the extruded eggs in the middle with its broken parts prolonged toward the cavity of the spermatheca (Fig. 4B, C, Fig. 5A). Figures 4D and E and 5B show that near the end of egg-laying, eggs are extruded continually. The split parts of the tissue are closely attached to each other when there are no eggs passing through.

One day after egg-laying. A new thin layer of valve-like tissue appears on the border that is connected to the epithelium of the spermatheca. The split parts of the valve-like tissue have already fused together. However, no nuclear-division is observed in this tissue. The old tis-

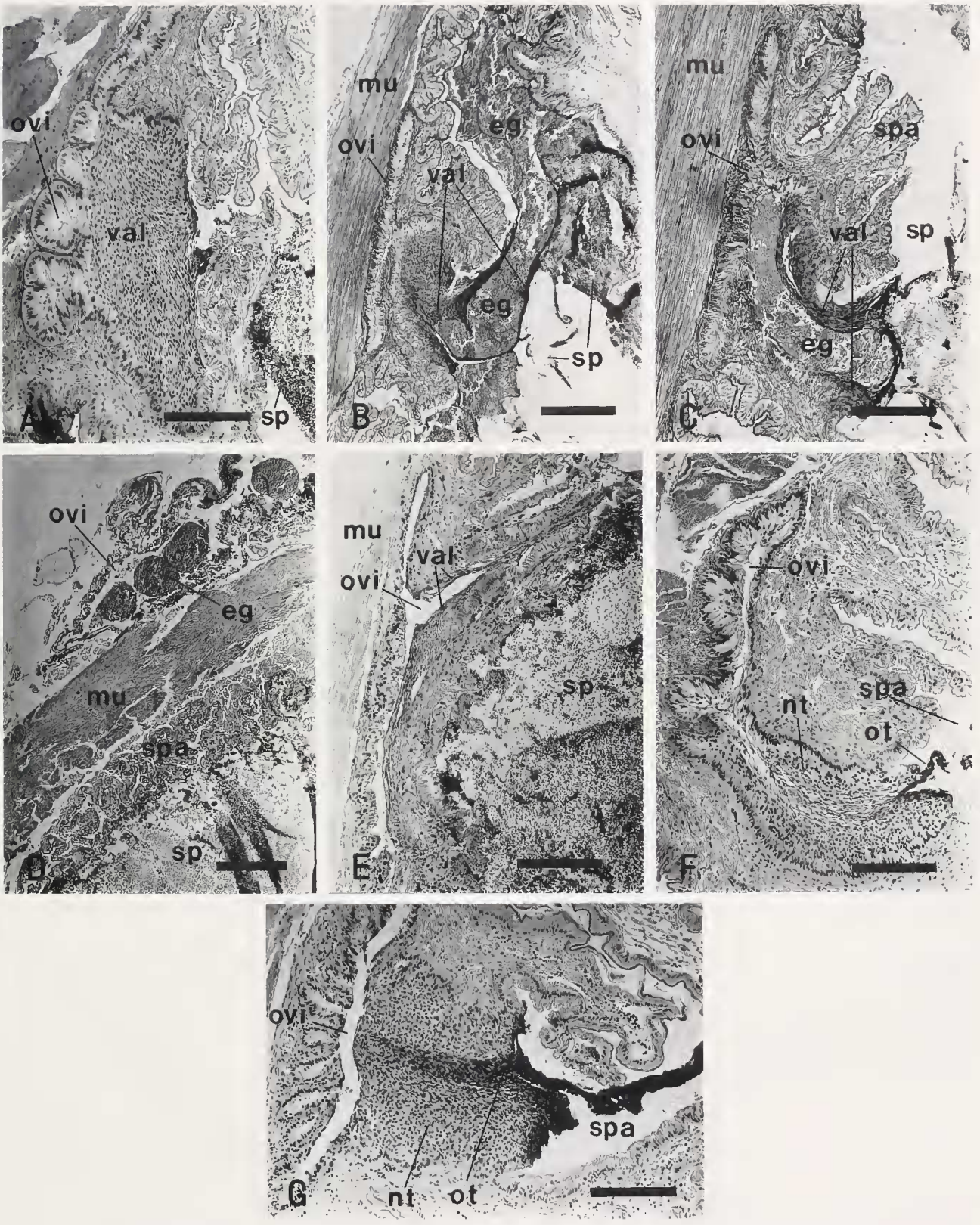

Figure 4. Transverse sections from the valve-like tissue and oviduct showing the structural changes in different stages. A: Five hours after copulation. B and C: During egg-laying (from the same spermatheca). D and E: Near the end of egg-laying (from the same spermatheca). F: One day after egg-laying. G: Four days after egg-laying. Abbreviations: (eg) egg; (mu) muscles; (nt) new valve-like tissue; (ot) old valve-like tissue; (ovi) oviduct; (sp) spermatozoa; (spa) spermatheca; (val) valve-like tissue. Bar (A, C, E, F, G) = 200 μm. Bar (B, D) = 400 μm.

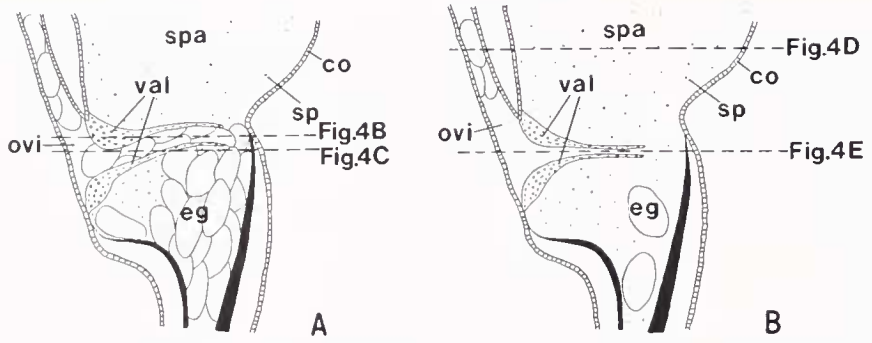

Figure 5. Schematic illustration of the longitudinal sections of the valve-like tissue. A: Based on observation of continuous transverse sections of the same spermatheca as shown in Figure 4B and C. B: Based on observation of continuous transverse sections of the same spermatheca as shown in Figure 4D and E. Abbreviations: (co) columnar epithelium; (eg) egg; (ovi) oviduct; (sp) spermatozoa; (spa) spermatheca; (val) valve-like tissue.

sue is being discharged into the cavity of the spermatheca. The surface of the oviduct changes again from being straight to undulant (Fig. 4F).

Four days after egg-laying. The old valve-like tissue is almost discharged and a new valve-like tissue is formed (Fig. 4G).

No spermatozoa were found inside the oviduct or the ovary during the different stages discussed above. Moreover, none of the ripe eggs removed from the egg-laying ovary developed into embryos.

Discussion

In the present study, no spermatozoa were found in either the oviduct or the ovary in *Eriocheir sinensis* before, during, or after egg-laying. Moreover, the ripe eggs removed from the egg-laying ovary did not develop into embryos. Therefore, the spermatozoa in the spermatheca never entered the oviduct or ovary. This phenomenon can be explained by the presence of the valve-like tissue. This tissue not only prevents the sperm from entering the oviduct or the ovary before egg-laying, but it also functions as a valve. It allows ripe eggs out of the oviduct during egg-laying and prevents the sperm from entering the oviduct, both near the end of egg-laying and after egg-laying, by closing once the positive pressure of the seminal fluid acts upon it (Fig. 6). Therefore, the only site where the eggs and sperm come into contact is in the spermatheca.

In *E. sinensis*, egg-laying continues for approximately 15–30 min. About 300,000–500,000 eggs or more can be found in one brood. The capacity of the spermatheca is no more than 100 eggs, and the opening of the vagina will only allow the passage of two or three eggs at one time. Thus, we estimate that the time an egg takes from entering the spermatheca to release from the vagina is no more than 1 s (unpub. data). Accordingly, there is only enough time for the sperm to attach to or penetrate the surface of the outer membrane of the ripe egg in the spermatheca, so the remaining events of fertilization must then occur externally. This conclusion is similar to the suggestions made by Yonge (1937), Ryan (1967), and Hinsch (1971).

Because fertilization is a series of phenomena that generally involves the contact of sperm and egg, penetration, and karyogamy, the term "internal fertilization" is apparently not appropriate for *E. sinensis*. However, in this study, we could not determine whether any interaction (*e.g.*, acrosome reaction) occurred between the sperm and the egg within the spermatheca. If such an interaction does occur, then fertilization in this crab cannot be external. On the other hand, if the sperm simply attaches to the egg membrane and has no further interaction with it within the spermatheca, then the term "external fertilization" is applicable. Hence, further research is required to determine whether this crab performs "external fertilization."

In early studies of the brachyurans by Binford (1913; *Menippe mercenaria*), Ryan (1967; *Portunus sanguinolentus*), and Hartnoll (1968; *Carcinus maenas*, *Hyas coarctatus* and *Hyas araneus*), the structure of the oviduct and its opening into the spermatheca were described as being similar to one another. Unlike *E. sinensis*, the oviducts of these crabs do not function as a passage from the ovary into the cavity of the spermatheca; rather, they are simply a convoluted cord of cells with a blind end extended toward the stratified epithelium of the spermatheca, except during ovulation (Fig. 7A). Some time before either ovulation or egg-laying, this cord of cells forms a passage between the ovary and the cavity of the spermatheca; but no valve-like tissue preventing the sperm from entering the oviduct was observed (Fig. 7B).

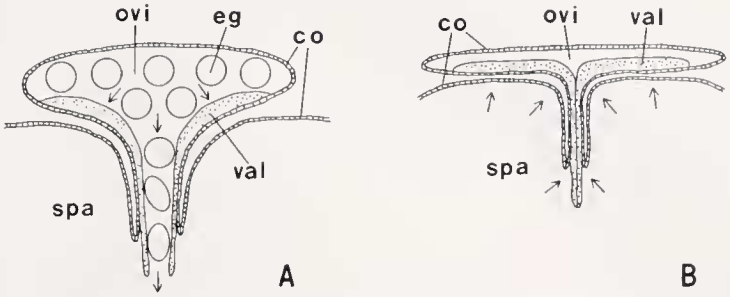

Figure 6. Schematic illustration of the transverse sections of the valve-like tissue showing the function of freeing eggs and preventing sperm from entering the oviduct. A: Valve-like tissue opens when the eggs are extruded. B: Valve-like tissue closes when the positive pressure of the seminal fluid acts upon it. Abbreviations: (co) columnar epithelium; (eg) egg; (ovi) oviduct; (spa) spermatheca; (val) valve-like tissue. Arrows indicate the directions of positive pressure.

Our view of the relation between the structural changes in the oviduct and the site of fertilization differs from those of previous investigators. Ryan (1967) found a small amount of sperm in the open oviduct of *P. sanguinolentus*. With no further explanation, he concluded that the spermatheca was the site of sperm-egg contact, and that the rest of the fertilization process occurred externally. He thought that there was insufficient time for the sperm to penetrate the egg within the body of the female crab. Diesel (1989) also reported sperm-egg contact within the spermatheca of *I. phalangium*, but did not report whether any sperm were present in the oviduct or ovary immediately before or after spawning. In studies of *M. mercenaria* (by Binford, 1913), and *C. maenas* (by Spalding, 1942; Cheung, 1966; Goudeau, 1982), the authors believed that fertilization occurred in the lumen of the ovary or within the oviduct because: (1) sperm were found in the lumen of the ovary; and (2) some of the eggs removed from that ovary could develop into embryos. We cannot deny that sperm might be naturally pressed into the oviduct and the lumen of the ovary when the blind-ended oviduct opens. However, the evidence cited by Binford (1913) and other investigators is too weak to support their conclusions. During their dissection and removal, the genital organs may have experienced negative pressure inside the ovary, drawing the sperm into the oviduct or the ovary. This artificial phenomenon might have misled investigators. This may also account for the presence of the sperm in the oviduct of *P. sanguinolentus* (by Ryan, 1967). Further experiments are needed to clarify the site of fertilization in the crabs that have no apparatus to prevent sperm from entering the oviduct.

There are two known types of oviducal openings into spermatheca: one is that reported by Binford (1913), Ryan (1967), and Hartnoll (1968), and the other is the one we describe in the present study. Besides *E. sinensis*, we also found the same valve-like tissue and patent oviduct in *Eriocheir japonicus* and *Hemigrapsus sanguineus* (unpub. data). In *Pachygrapsus crassipes*, Chiba and Honma (1971) discovered an oviduct of the same structure; unfortunately, they did not mention whether there was a valve-like tissue. What does the distribution of these two types of openings in the Brachyura mean? What is their taxonomic significance? Does the valve-like tissue have functions other than preventing sperm from entering the oviduct? From where are the cells that rebuild the split valve-like tissue?

The presence of the valve-like tissue in crabs helps ensure that the ripe eggs removed from the egg-laying ovary are all unfertilized. This finding is of importance for obtaining unfertilized ripe eggs in studies of artificial fertilization (*in vitro*) (Lee and Yamazaki, 1989) in *E. sinensis*. Furthermore, crabs with this valve-like tissue would be good laboratory animals for studies on fertilization, hybridization, and embryology in the Brachyura.

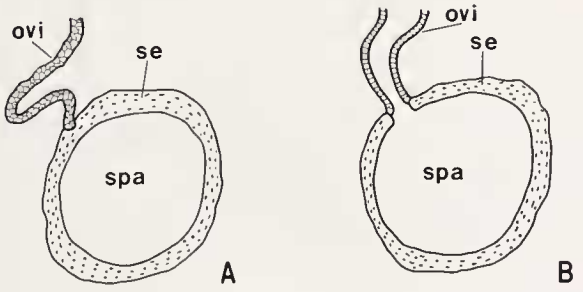

Figure 7. Schematic illustration of the transverse sections of the oviduct and spermatheca described in early studies by Binford (1913), Ryan (1967), and Hartnoll (1968). A: Non-spawning stage. B: Some time before or during ovulation and egg-laying. Abbreviations: (ovi) oviduct; (se) stratified epithelium; (spa) spermatheca.

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